Protocol for the Assessment of Potential Health Effects From Embedded Metal Fragments

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ABSTRACT Potential health issues arising from embedded metal fragment injuries are an operational health issue in the military medical community. Embedded fragment injuries can occur not only from standard-use munitions, but also from improvised explosive devices (IEDs). With few exceptions, the behavior of metal fragments, when embedded in the body, is not known. Herein, we propose a multitiered assessment protocol that can be used to both determine future health issues associated with newly developed munitions and, once the composition has been identified, determine potential toxicity of embedded fragments as a result of an IED event. The main premise of this assessment protocol is to view the embedded fragment as an implanted medical device and to utilize the guidelines already in place for testing the safety of such materials. Use of this structured, tiered approach will yield information critical for informed medical decisions.

INTRODUCTION

Many unique metal mixtures are found on today's battlefield, both in newly developed munitions as well as in improvised explosive devices (IEDs). Because of the lack of relevant data, the potential health effects of embedded metal fragments from wounds with these materials are not known. Such a situation puts health care professionals at a disadvantage when treating injuries of this type. There is a clear need within the military medical community to characterize the toxicological properties of embedded metal fragments and to have that information readily available to health care professionals. One has only to look at depleted uranium (DU) and heavy-metal tungsten alloy (WA) to realize that this type of testing is not currently done.

The first widescale use of DU munitions was in the First Gulf War where several friendly fire incidents resulted in a number of Coalition personnel being wounded by DU fragments.[1] Although the existing literature at that time provided an appraisal of DU hazards via inhalation or ingestion, little was known about the health effects of DU exposure because of wound contamination or embedded fragments. As a result of this lack of information, several research projects were undertaken to investigate the long-term health effects of embedded DU fragments.[2,3] However, these were not initiated until after concern was raised about the long-term impacts of embedded DU fragments. As a result of widespread concern over the use of DU, alternative materials were sought and some heavy-metal tungsten alloys were identified as likely replacements, but these materials were not tested early in the munitions development process for adverse health effects as embedded fragments. A recent report describing the induction of a highly aggressive rhabdomyosarcoma in rats implanted with a military-grade tungsten/nickel/cobalt alloy raises serious questions as to the health effects of these compounds.[4]

The lack of information on potential toxicity of embedded metal fragments resulting from wounds inflicted by standard munitions is disconcerting. The incorporation of simple basic tests for corrosion/dissolution and cytotoxicity would not only supply information critical in formulating treatment policies, but, if incorporated at a point early in the munition development process, could provide weapon developers and risk assessors the data required to make informed decisions as to whether to continue with a particular metal mixture or seek an alternative. Such decisions made early in the development process would ultimately save considerable time and money. Department of Defense (DoD) policy requires that the health effects of new munition systems be evaluated.[5–8] However, most analyses only investigate likely exposures to the system operator, not the potential of embedded metal fragments.

Another area of concern is the continued use of IEDs by terrorists and insurgents in both Iraq and Afghanistan. In Iraq, through September 2007, IEDs were responsible for 80% of all military casualties.[9] Although many of the embedded metal fragments are a result of the bomb itself, some are from the components and shielding of the destroyed vehicle. The recently released DoD Health Affairs policy on analysis of excised fragments dictates that all removed fragments be analyzed for chemical composition.[10] With this information, a determination of the source of the metal fragment (bomb component or vehicle part) could likely be made. Regardless of the original source of the fragment, information on its potential health effects would be valuable. However, vehicle metals likely to become embedded as fragments as a result of an IED could be tested for potential adverse health effects and that information made available to medical personnel. Metal

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fragments not linked to a vehicle component would have probably come from the IED itself. Although the list of possible metals in an IED might seem endless, common battlefield material such as unused munitions, stainless steel, and iron would probably be the most likely candidates. Again, metal analysis of excised fragments would be invaluable in this regard.

What will not be answered by fragment metal analysis are the potential health effects of the remaining embedded fragments. Standard surgical guidance recommends leaving embedded fragments in place unless they can be easily removed, are in a joint or near a vital organ, or will cause future health issues. Aggressive surgical removal must be tempered by the goal to minimize collateral muscle damage when attempting to remove fragments. Clearly, knowledge of the potential health effects of a specific metal compound, when embedded as a fragment, would influence surgical removal policies. Therefore, what is proposed here is a comprehensive multitiered assessment protocol for determining the potential health effects of embedded metal fragments.

TABLE I. Screening Protocol for Assessing Potential Health Effects of Embedded Fragments

Tier 1 Testing
Corrosion Testing
ASTM Standards F746-04 and F2129-06
Cytotoxicity Testing
ISO 10993-5
Testing of additional cell lines using standard viability tests
Tier 2 Testing
Short-term Rodent Models (0–12 months)
Assessment of systemic toxicity (body and organ weights)
Localized tissue changes around fragment
Histopathology
Metal determination in body fluids and tissue
Hematological and serum chemistry assessments
Tier 3 Testing
National Toxicology Program Two-Year Study
Toxicity and carcinogenicity assessment
Specialized Assessments
Immunotoxicity
Neurotoxicity
Reproductive toxicity

Assessment Protocol

For the most part, embedded metal fragment wounds are a uniquely military injury. As a result, with the exception of the published studies on DU and WA, there is little in the literature concerning the long-term health effects of this type of exposure. However, if we view embedded metal fragments as an implanted medical device, we can utilize the established testing guidelines already available for determining the safety of those materials. In addition, there is a wealth of health and toxicity information available on medical alloys that may prove relevant for embedded fragment wounds.

The health effects assessment protocol is a three-tiered screening procedure that incorporates corrosion/dissolution determinations, in vitro cytotoxicity evaluations, and short- and long-term in vivo studies using rodent model systems (Table I). In many cases, not all testing tiers would be required depending on what preliminary information was available and what type of material was being tested. For example, new munition materials should undergo more extensive health and toxicity testing than would metal compounds only expected to be found embedded as a result of an IED event. Metals, and their alloys, for munitions or vehicle components that may end up as embedded fragments can be "prescreened" before use. Fragments analyzed after surgical excision and not previously tested can be "postscreened" using test material of similar composition. The protocol is easily expandable to allow the collection of information on other types of exposure scenarios, such as inhalation or ingestion, or additional areas of concern (e.g., immunotoxicity, neurotoxicity, reproductive toxicity, etc.).

Tier 1 Testing

This tier consists of two testing subgroups: corrosion or "dissolution" testing and in vitro cytotoxicity assessments. The ability of the metal compound to corrode or dissolve in biological fluids is an important first test in determining potential adverse health effects and metal bioavailability. The American Society for Testing and Materials (ASTM) has two standard test methods for determining corrosion susceptibility in medical implant devices that could be applied to metal fragment testing.[11,12] Both procedures use potentiometric polarizing techniques to artificially stimulate corrosion of the test material in a simulated physiological solution (phosphate buffered saline, Hanks' balanced salt solution, Ringer's solution). The procedures are designed to produce conditions severe enough to induce corrosion of the test material. Thus it is important to compare the results to a standard test material such as Type 316L stainless steel, an alloy considered acceptable for use in medical implants. It should be noted that the conditions specified in the testing protocols are of such a severity as to corrode Type 316L stainless steel, and, as such, may not be attainable in an in vivo situation. Therefore the results should be considered a "worst case" scenario. Much of the guidance in the ASTM standards is also included in ISO 10993-15.[13]

Most embedded metal fragments would probably not be composed of alloys used in medical devices and would be expected to corrode at a far faster rate when assessed by the ASTM standards. Additional information on stability could be obtained by testing fragment dissolution characteristics in synthetic biofluids designed to mimic the in vivo condition. Metal degradation in synthetic interstitial fluid,[14] lymph,[15] and synovial fluid[16] formulations should be examined to determine the extent of breakdown over time. The extractant should be analyzed for metal content using an appropriate analytical technique such as inductively coupled-plasma mass spectrometry. These data will give an indication of in vivo metal bioavailability. Although this protocol focuses on embedded fragment toxicity, the tests listed here can easily be expanded to address other exposure scenarios. For

example, depending upon the end use of the metal alloy and potential exposure scenarios, degradation testing in other synthetic body fluids (lung,[17] gastric,[18] serum[19]) could be applicable. Standard procedures for assessing the solubilization of metals and alloys in biofluids are readily available and are well documented in the review of Ansoborlo et al.[20]

The second section in this tier assesses cytotoxicity using in vitro methodology. At a minimum the guidance in ISO10993-5 for determining cytotoxicity should be followed.[21] This standard determines the cytotoxicity of material on cultured cell lines. Materials are tested for effects as a result of direct exposure to the cells and after extraction in culture medium. Cytotoxicity is determined by microscopic examination of the cells and an assessment of morphology, detachment from the plate, vacuolization, and cell lysis. Unless available in the literature, it would also be useful to test the individual metals comprising the fragment for cytotoxicity. This should be done with both soluble and insoluble forms of the metals. These results will provide the basis for estimating potential toxicity on the basis of the initial fragment composition analysis. A variety of cell lines that represent tissues that are common targets of metal toxicity in the body should be utilized for testing. As previously noted, although this protocol deals primarily with embedded fragments, it can easily be expanded to test potential toxicities associated with other exposure scenarios. The cell lines to be utilized will, of course, depend upon any additional exposure scenarios investigated. Table II gives a list of some of the cell lines used in our studies on metal toxicity. The list is by no means comprehensive. The American Type Culture Collection designation is provided as a convenient source for additional information and does not constitute an endorsement by us or the Department of Defense. All cell lines should come from reputable vendors and tested before use to eliminate questions of cross-contamination and authenticity.[22] Although qualitative assessment by microscopic examination should be done (ISO 10993-5), cell viability assessments to indicate not only the cytotoxic potential of the metal, but also the possible site of damage would be useful. Several of these tests are listed in Table III. Again the list is not intended to be inclusive, but rather an overview of the types of assays available. The assays can be loosely grouped into tests of membrane damage, cell proliferation capacity, and assessments of metabolic viability. Selection of a particular assay will depend on individual laboratory capabilities and expertise. At a minimum, one test from each of the three categories should be selected to provide an adequate determination of the cytotoxic potential of the material. The results obtained from corrosion and dissolution testing, as well as cytotoxicity assessments, will determine whether the material should be screened at a tier 2 level.

TABLE II. Cell Lines for In Vitro Cytotoxicity Screening

Cell Line	ATCC Designation	Species	Tissue	Cell Type
NCTC clone 929	CCL-1	Mouse	Connective	Fibroblast
C2C12	CRL-1772	Mouse	Muscle	Myoblast
L6	CRL-1458	Rat	Muscle	Myoblast
J774A.1	TIB-67	Mouse	Ascites	Macrophage
Reh	CRL-8286	Human	Blood	Lymphoblast (B-cell)
Molt-4	CRL-1582	Human	Blood	Lymphoblast (T-cell)
HepG2	HB-8065	Human	Liver	Epithelial
LLC-PK1	CL-101	Pig	Kidney	Epithelial
A549	CCL-185	Human	Lung	Epithelial
Caco-2	HTB-37	Human	Colon	Epithelial
N1E-115	CRL-2263	Mouse	Brain	Neuroblast

Tier 2 Testing

Materials determined to be corrosion prone and cytotoxic as a result of tier 1 testing, or those in widescale use, should be assessed using tier 2 protocols. The testing proposed in tier 2 involves short-term (0–12 months) in vivo testing of embedded fragments in a rodent model system. The guidelines set forth in ISO 10993-11 should be used as a framework for designing the in vivo studies.[23] Mice or rats are the model system of choice and a list of commonly used strains are given in Table IV. A protocol for the surgical implantation of fragments has been developed and validated for embedded DU and WA fragments.[24] If properly conducted, a tremendous amount of information can be gleaned from these types of studies.

Experimental groups should encompass short-term, mid-range, and long-term exposure periods. Thus, groups implanted for 1, 3, 6, and 12 months are most reasonable. It is also vital to run the proper control groups to validate the data obtained. As with tier 1 testing, this section can also be expanded to include additional assessments. Basic information that should be collected includes weekly body weights and an evaluation of the general health of the animal. Body weight has been shown to be a sensitive indicator of systemic toxicity. In addition, the fragment implantation sites should be examined at least weekly and the region manually palpitated to detect abnormal growth. Upon euthanasia, the implanted fragments should be removed and examined for corrosion and the surrounding muscle inspected for abnormalities by histopathology.

TABLE III. Tests of Cell Viability

Membrane Damage Tests
Trypan blue dye exclusion
Lactate dehydrogenase release
Propidium iodide uptake
Cell Proliferation Determination
Cell counting
Total cellular protein determination
Colony formation capacity
Metabolic Viability Assays
MTT conversion
Alamar blue reduction
Neutral red retention

TABLE IV. Rat and Mouse Strains for Toxicity Testing

Species	Strain	Genome
Mouse	CD-1	Outbred
Mouse	Balb/c	Inbred
Mouse	C3H	Inbred
Mouse	C57BL/6	Inbred
Mouse	B6C3F1	Hybrid
Mouse	CD2F1	Hybrid
Rat	Sprague-Dawley	Outbred
Rat	Fischer 344	Inbred
Rat	Wistar-Han	Outbred

Organs should be removed, weighed, and submitted for histopathology examination and metal analysis. Blood and urine should also be collected. A complete hematological workup, as well as serum chemistries, should be done. Urine and serum should also be analyzed for metal content. Subtle immune system changes can be determined by using flow cytometry techniques to determine immune cell distribution patterns in peripheral blood, thymus, spleen, and bone marrow.

The results of tier 2 testing will provide in vivo data on the local and systemic toxicity of the embedded fragment. Examination of the excised pellets will give an indication of corrosion potential, while metal analysis of the tissues and body fluids will show bioavailability and target organs for the solubilized metals. Especially important are the serum and urine metal measurements, as significantly elevated metal levels in those samples may provide a means for determining the composition of embedded fragments that are not surgically removed.

Tier 3 Testing

Depending upon the results obtained from tier 1 and 2 testing, a decision may be made to continue to tier 3. In many cases this will not be necessary since sufficient information as to the potential toxicity of a particular metal formulation will probably be evident from the tier 1 and 2 data. Tier 3 testing follows the National Toxicology Program (NTP) guidelines for determining toxicity and carcinogenicity.[25] These investigations use specific rodent models in a 2-year life span study. Currently, the B6C3F1 mouse and the Wistar-Han rat are the strains of choice of the NTP. Fragments would be surgically implanted as described in tier 2 testing. NTP guidelines recommend testing both sexes at three different experimental doses with 50 animals per dose. After adding suitable control groups to the experimental design, it is easy to see that these tests are not only lengthy, but can also be expensive to perform. However, they are currently considered the "gold standard" for assessing potential toxic and carcinogenic effects. Tier 3 would also encompass more specialized testing that may be required such as immunotoxicity, neurotoxicity, and reproductive toxicity screening. Guidelines for conducting such studies have been developed by the United States Environmental Protection Agency.[26] In many cases, they can be included as part of a NTP life span study.

CONCLUSIONS

The lack of information on the toxicological properties of embedded fragments from newly developed munitions or IEDs has put health care professionals at a disadvantage when treating these types of wounds. Standard surgical procedure dictates that fragments are to be left in place unless they can easily be removed, are in a joint or near a vital organ, or will cause health issues in the future. But medical personnel cannot make informed decisions about future health issues if no information is available on the toxicological properties of the embedded metals. Therefore, we have proposed a screening protocol for assessing the potential health effects of embedded fragments that uses a tiered approach for testing the toxicological properties of embedded fragments. This protocol only describes a framework within which materials can be tested. Before initiation of any assessment scheme, all testing guidelines should be harmonized so that valid interlaboratory comparisons can be made. Testing of new munitions should be undertaken when these materials are still in the developmental phase. Testing of embedded fragments will either have to await surgical excision and analysis as mandated by Health Affairs Policy 07-029 or, more proactively, compositions similar to those already excised and analyzed can be assessed. Not only should potential IED material be assessed, but those vehicle components that have already been identified in fragment wounds or that are likely to become an embedded fragment as a result of an explosion should be screened. The early assessments can easily be expanded to address other exposure scenarios such as inhalation and ingestion. The extent of testing is only limited by funding constraints, but clearly any material expected, or already found, as embedded fragments, should be tested at a tier 1 level and possibly tier 2.

Not every metal compound in use today can be tested for adverse health effects. Rational selection of those compounds likely to be found as embedded fragments would greatly reduce the list of materials requiring assessment. Cost is often cited as a deterrent to comprehensive testing. However, although tier 3 testing can be expensive, tier 1 and 2 assessments, in most cases, will provide sufficient information to make informed decisions on treatment options. In addition, when considered in the context of the overall cost of weapon systems development, the price of a comprehensive health effects testing protocol is miniscule. If included early in the process, such screening has the potential to uncover any unanticipated health issues that could force abandonment of the system in the future, well before a significant investment in time and money has already been made.

The ultimate goal of the proposed screening protocol is the development of a database containing the results of the toxicity testing, as well as any information culled from the scientific literature on previously conducted studies. In this way, testing redundancy can be avoided. Access to the database for all Department of Defense, as well as Department of Veterans Affairs, medical personnel is critical for the information to reach those who need it. Only by understanding the potential toxicological effects of embedded fragments can sound treatment decisions be made. Technological advances have

made wounds with embedded fragments of unique metal compounds an urgent concern for those in the military medical community. We owe it to our wounded soldiers to provide the best care possible. Development of a database of toxicological properties of embedded metal fragments will provide one of the tools required to do this.

To summarize the key points of the proposed screening protocol for embedded fragments:

- For testing purposes, embedded fragments should be considered as an implantable medical device and the available standardized testing guidelines for corrosion, dissolution, and toxicity employed.
- Any material expected to be found embedded as fragments as a result of normal munition use or from an IED event should, at a minimum, be tested at a tier 1 level.
- New munition material should be tested at both tier 1 and tier 2 levels. Results from those assessments will determine whether tier 3 testing is warranted. This testing should be done as early in the development process as is feasible.
- Results from studies on the toxicity of embedded fragments should be located in a database readily accessible to health care professionals.

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